Transformation of *HBsAg* (Hepatitis B Surface Antigen) Gene into Tomato Mediated by *Agrobacterium tumefaciens*

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Abstract: The plant expression vector pBRSAg was constructed as suitable for transformation via *Agrobacterium*-mediated approach. It contains all elements for plant expression, such as CaMV 35S promoter, both left and right border sequence for transferred DNA (T-DNA) in *Agrobacterium*, plant reporter gene *gus*, and plant selection marker gene *hpt*. The recombinant binary vector pBRSAg was transformed into *Agrobacterium tumefaciens* strains by using the freeze-thaw method. Tomato cotyledon explants were transformed by *A. tumefaciens* and plants were regenerated on selection medium. GUS staining, PCR and PCR-Southern analysis of the plants were positive. It was for the first time shown that the *HBsAg* (hepatitis B surface antigen) gene was introduced into tomato plants. The expression of transgene is under investigation.

Keywords: *Agrobacterium*; *HBsAg* gene; tomato; transformation

With the development of gene engineering technology, the study of using gene modified plants to produce vaccines and medications has become a hot spot. Plant-based vaccines have attracted the attention of worldwide scholars for its safety and economy, most of which have been expressed through genetic transformations and have shown satisfactory effects and have been put into commercial production, such as the vaccines of Hepatitis B (Mason *et al*. 1992), pulmonary tuberculosis (Rigano *et al*. 2006), rabies (Modelska *et al*. 1998), measles virus (Bouche *et al*. 2003) *etc*.

Hepatitis B virus (HBV) is a global disease. It can be transmitted by blood and body fluid and its pathogenesis is complex. With the increasing infection of HBV, it heavily threatens people's health. Epidemiological data reveal that there are about 360 million carriers of hepatitis B virus throughout the globe and 78% of the world populations' hail from Asia (Behal *et al*. 2008). Nowadays, an injection of HBV vaccine is the only economic and effective way to control this disease. The commercially available HBV vaccine is yeast-derived and the high cost has led to the research for cheaper and safer methods to produce effective hepatitis B vaccines (Higashihashi *et al*. 1991; Schodel *et al*. 1994).

Although a vast amount of information is available about the gene transformation of hepatitis B surface antigen (*HBsAg*), there are still many truths to seek. Successful treatment of HBV carriers with HBsAg plant vaccine from plants must have a beneficial effect on regions without favourable environmental sanitation.

Genetic engineering can be used to produce desirable agronomic traits quickly and efficiently, and also to introduce genes encoding high-value recombinant proteins (Arokiaraj *et al*. 2002). In comparison with the traditional expression systems, plant-derived hepatitis B vaccine has attracted the attention of the pharmaceutical industry for reasons of safety and economy (Walmsley &

Arntzen 2000). In the experiment with mice and human beings, parenteral immunization of plant-derived hepatitis B surface antigen stimulated B and T cell immune responses similar to those found with commercial yeast-derived hepatitis B vaccine (Thanavala *et al.* 1995). So, it is feasible to produce hepatitis B vaccine via transgenic plants.

The objective of presented study was to transform tomato – as a candidate crop for edible vaccine production – with *HBsAg* gene via *Agrobacterium*-mediated approach.

MATERIALS AND METHODS

Plant material

The seeds of tomato Yuguang 401 were purchased from the Academy of Agricultural Sciences in Fujian Province. The seeds were surface sterilized with 75% alcohol for 40–60 s and washed 3–4 times in sterilized distilled water. Then the seeds were sterilized with 2% NaClO for 10 min and washed again. Finally, the seeds were sown and germinated on half-strength Murashige and Skoog (1962) (MS) medium. The cotyledons of germinated seedlings were isolated, their apical parts were removed and cotyledons were sectioned transversely into two segments.

Construction of plant expression vectors

Plasmids pSPROK, pCAMBIA-1301, and pBSAg were preserved in our laboratory, of which the plasmid pSPROK contains CaMV35s promoter and nos terminator, plasmid pCAMBIA-1301 contains hyg-resistance gene, kan-resistance gene and *gus* gene, and plasmid pBSAg contains the 681 bp DNA fragment (named HBsAg).

The fragment containing P35s-Tnos was isolated by gel extraction from plasmid pSPROK after *Eco*R I and *Hin*d III restrictive digestion and inserted into plasmid pCAMBIA-1301, which had been digested by the same restriction endonuclease to yield the reconstructed plasmid pCBROK. A 681 bp DNA fragment named HBsAg was obtained by gel extraction from plasmid pBsAg after *Xba* I and *Kpn* I restrictive digestion and then subcloned into plasmid pCBROK, which had been digested by the same restriction endonuclease to yield the reconstructed plant binary expression plasmid pBRSAg (Figure 1). All plasmids were isolated by the "alkaline lysis method" and purified by phenol and chloroform extraction (Sambrook *et al.* 1989).

Agrobacterium strain

The *A. tumefaciens* strains LBA4404, EHA105 and C58 were transformed with the recombinant binary vector pBRSAg via the freeze-thaw method (Hofgen & Willmitzer 1988). Transformants were selected on LB agar (casein enzymic hydrolysate 10 mg/l, yeast extract 5 mg/l, sodium chloride 5 mg/l) plate containing kanamycin 50 mg/l (Sigma, St. Louis, USA), rifampicin 50 mg/l and streptomycin 200 mg/l (Shandong Xinhua Pharmaceutical Company, Zibo, China)and the resistant colonies were verified by restriction enzyme digestion and used in transformation experiments.

Agrobacterium mediated transformation and multiplication of transgenic plants

Tomato cotyledon explants were pre-cultivated on MS medium with 6-BA 2.0 mg/l and IAA 0.2 mg/l (Shanghai Chemical Reagent Company, Shanghai, China) for 2 days, then they were transformed by *A. tumefaciens* with pBRSAg. The cotyledon explants after co-cultivation for 2 days on MS with 6-BA

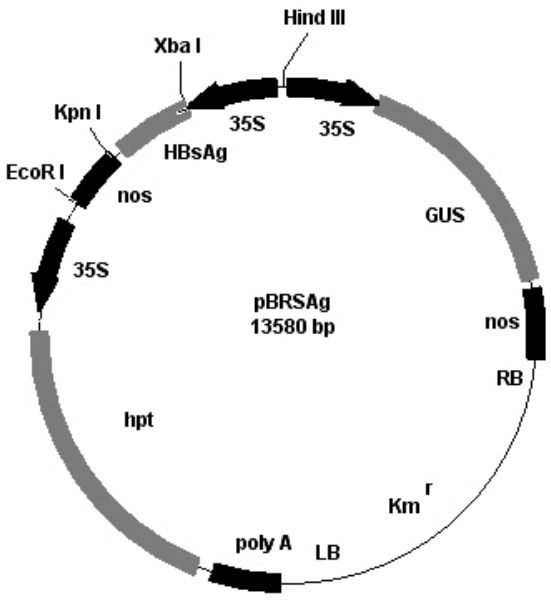

Figure 1. Structure of plasmid pBRSAg

2.0 mg/l and IAA 0.2 mg/l were transferred to MS selection medium with 6-BA 2.0 mg/l, IAA 0.2 mg/l, timentin 200 mg/l (SmithKline Beecham Pharmaceuticals, Worthing, UK) and hygromycin 7 mg/l (Sigma, St. louis, USA). Shoots were generated from the transformed callus on selection medium after 4–5 weeks. The shoots which grew into 2–3 cm were transferred for rooting on MS with IAA 0.5 mg/l, timentin 200 mg/l and hygromycin 7 mg/l. The rooting plants growing into plantlets were transplanted into soil.

GUS staining

Both transformed and untransformed leaves of tomato plants were immersed into GUS reaction buffer for 12 to 24 h at 37°C and then bleached with absolute alcohol for 3–5 h.

Molecular analysis

PCR analysis. The modified method of CTAB (Stewart & Via 1993) was used to extract the genomic DNA of transgenic tomato plants and untransformed tomato plants. PCR method was used to confirm the transgene integration into the GUS positive putative transformants. The primer pairs were: forward: 5' -CGCAGTCCCAAATCTCC-3', and reverse: 5'-TGGTAACAGCGGCATAAA-3'. 20 ml of PCR mix contained 250 ng of genomic DNA as template, 20mM of each primer, 0.3 ml of Taq DNA polymerase (5 U/ml) (Takara, Dalian, China), 1.8 ml of each dNTP (2.5mM), 2.0 ml of 10 × PCR buffer. Cycling parameters were at 94°C for 6 min, followed by 35 cycles at 94°C for 1 min, 52°C for 1 min, and at 72°C for 1 min, and a final extension at 72°C for 10 min, 4°C preservation.

Southern analysis of PCR products. PCR-Southern blotting was carried out to further verify the integration of the target gene (*HBsAg*) into the genome of tomato plants. The blotting and subsequent hybridization were carried out according to Sambrook *et al*. (1989).

Statistical analysis

All data on the transformation frequency represent the mean values of three independent experiments with a minimum of 50 explants per treatment.

RESULTS AND DISCUSSION

Effects of major factors on tomato transformation

The genetic transformation mediated by *Agrobacterium* is a complicated process which is influenced by many parameters such as *Agrobacterium* strain (Crane *et al*. 2006), explant genotype (Chen *et al*. 2008), co-cultivation duration (Barik *et al*. 2005) and signal molecules (Satchel *et al*. 1985). High-frequency transformation relies on the highly efficient T-DNA delivery from *Agrobacterium* into plant cells (Ko *et al*. 2003).

Different strains of *A. tumefaciens* vary in their transforming abilities. A comparative study was performed on the interaction of three different *A. tumefaciens* strains (LBA4404, EHA105 and C58). According to the results of the GUS expression frequency, *A. tumefaciens* strain LBA4404 was found to be the most suitable, as GUS expression was the highest (21%) in these explants when compared to EHA105 and C58 (Figure 2A). So *A. tumefaciens* strain LBA4404 was used in all subsequent experiments. The results were in line with those of Bhattacharjee *et al*. (2010). In their study, three different *Agrobacterium* strains (LBA4404, EHA105 and AGL1) were used and the highest number of blue-stained tissue activity was obtained from the putative transgenic tissue infected by LBA4404 strain.

Figure 2B shows a comparison of the transformation frequency after different infection time. The transformation frequency was increased with the infection time increasing from 0 to 10 min. Results showed that the 10-min infection time in the presence of *Agrobacterium* produced the highest percentage of GUS positive explants (19.6%). However, bacteria grew on the selection medium significantly at the time beyond 10 min and the transformation frequency was critically reduced. The results indicated that too short infection time was not favourable for transformation. However, too long infection time resulted in the overgrowth of *Agrobacterium* and therefore it was harmful to explants (Shivani *et al*. 2010).

Bacterial concentration plays an important role in the production of transformants. More necrosis of explants was seen at the highest concentrations of bacteria for all cultivars. There was a significant linear relationship between the percentage of necrotic explants and bacterial concentration,

and there was a significant interaction of cultivar with bacterial concentration (Davis *et al*. 1991). Our results showed that the transformation frequency was increased significantly as the bacterial concentration rose from 0.1 to 0.4 (OD_{600}), while the transformation frequency was reduced as the bacterial concentration rose from 0.6 to 1.0 (OD_{600}) (Figure 2C). The results were similar to those of Saini and Jaiwal (2007), who reported that higher *Agrobacterium* concentrations could

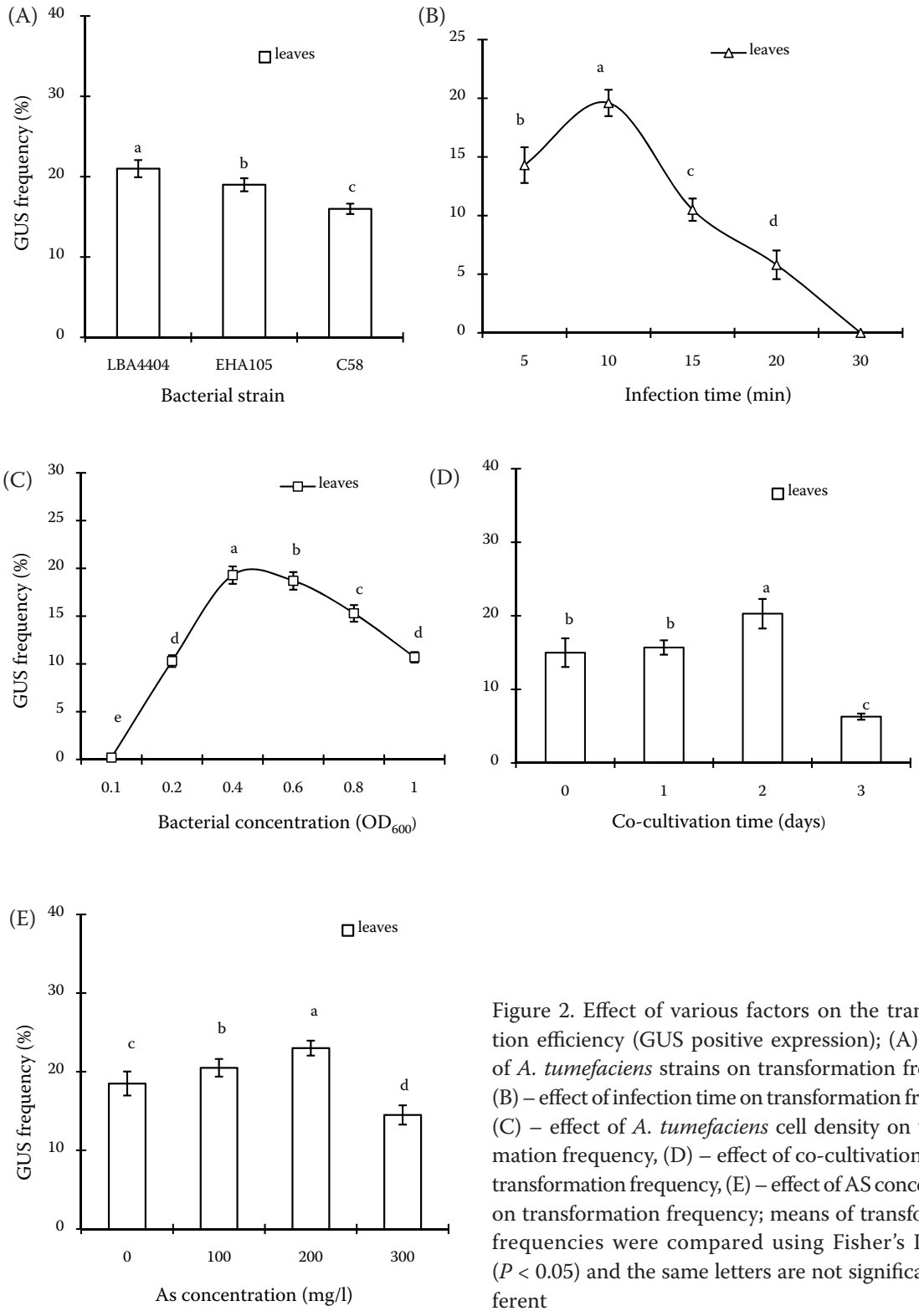

Figure 2. Effect of various factors on the transformation efficiency (GUS positive expression); (A) – effect of *A. tumefaciens* strains on transformation frequency, (B) – effect of infection time on transformation frequency, (C) – effect of *A. tumefaciens* cell density on transformation frequency, (D) – effect of co-cultivation time on transformation frequency, (E) – effect of AS concentration on transformation frequency; means of transformation frequencies were compared using Fisher's LSD test ($P < 0.05$) and the same letters are not significantly different

lead to hypersensitive responses of explants. Thus, a relatively lower inoculum density improved the transformation efficiency and reduced the *Agrobacterium* overgrowth in this study.

Co-cultivation is very important in the transformation process. Bacteria attachment, T-DNA transfer and integration were carried out during this stage, which could be accelerated by supplementing some ingredients to the co-cultivation medium or prolonging co-cultivation so that it could be terminated successfully (Barik *et al*. 2005). The influence of co-cultivation on *Agrobacterium*-mediated transformation has been reported in a number of plant species (Banerjee *et al*. 2002; Mohan & Krishnamurthy 2003). In our study, the highest GUS expression frequency was observed with 2-day co-cultivation and it was remarkably different from the other co-culture duration (Figure 2D). Two days were also confirmed as the optimum co-cultivation duration whereas 3-day co-cultivation might cause the overgrowth of *A. tumefaciens* leading to damage of the plant cells and consequently resulting in a low transformation frequency. On the other hand, a shorter co-cultivation time might disrupt *A. tumefaciens* cell proliferation, thereby reducing its virulence and leading to a low transformation frequency.

Some recalcitrant plant species can be transformed by inducing the vir genes of the bacteria by signal molecules or *in vitro* by co-cultivation of *Agrobacterium* with wounded tissue or in media that contain signal molecules (Satchel *et al*.1985). Acetosyringone (As) or related compounds functioning as signal molecules have been reported to improve the *Agrobacterium*-mediated transformation in several plant species (Švábová & Griga 2008; Wang *et al*. 2009). In our study, 100 mg/l and 200 mg/l As significantly improved tomato transformation efficiency, while 300 mg/l As significantly reduced tomato transformation efficiency, the maximum frequency of transformation was observed at 200 mg/l As (Figure 2E). Our experiments showed that tomato transformation efficiency was enhanced under low As concentration, but it was reduced under high As concentration. The present results were in line with those of Priya & Shivendra (2009).

Transformed plants were selected on the basis of their resistance to hygromycin. The integration of transgene in different lines was confirmed by GUS staining. Taken together, tomato shoot production under the optimal conditions determined above was conducted: strain LBA4404, 0.4 bacterial concentration (OD_{600}), 10-minute infection, co-cultivation for 2 days and 200 mg/l acetosyringone.

Regeneration and GUS staining of transgenic plants

Transformed tomato cotyledon explants showed the development of shoots on the hygromycin selection medium. The shoots were excised and cultured on a rooting medium with 7 mg/l hygromycin. The transformed shoots could develop roots and grow into complete plantlets on the selection medium of this concentration whereas untransformed shoots hardly developed any roots and hardly grew into plantlets. The shoots were transferred to a new selection medium every two weeks. Most untransformed shoots were eliminated during about 4–6 weeks of selection and rooting culture. Twenty-one independent hyg-resistant plantlets were obtained after about 2 months of selection (Figure 3).

The transformation events of putative hygromycin-resistant transgenic lines were confirmed by GUS histochemical analysis. After tomato leaf tissues were stained with GUS reaction solution and bleached with absolute alcohol, the whole of the transformed tissues appeared nearly deep blue colour while untransformed tissues appeared colourless. GUS histochemical assay is a convenient and fast technique to detect the integration and expression of target gene which was widely used in many plant species (Švábová *et al*. 2005; Sri Shilpa *et al*. 2010). The GUS histochemical assay of the leaves of T_0 plants showed blue colouration, confirming the presence of the transgene (Figure 3F). In this study, we used stringent hygromycin selection, so GUS expression was observed in most leaves of tomato cultivars. This result also indicated the reliability of the selection regime for obtaining transformed tomato plants.

Molecular analysis of transgenic tomato plants

PCR was used to confirm the transgene integration into the GUS positive putative transformants. The partial gene-specific HBsAg fragment (495 bp) of the expected size was observed in

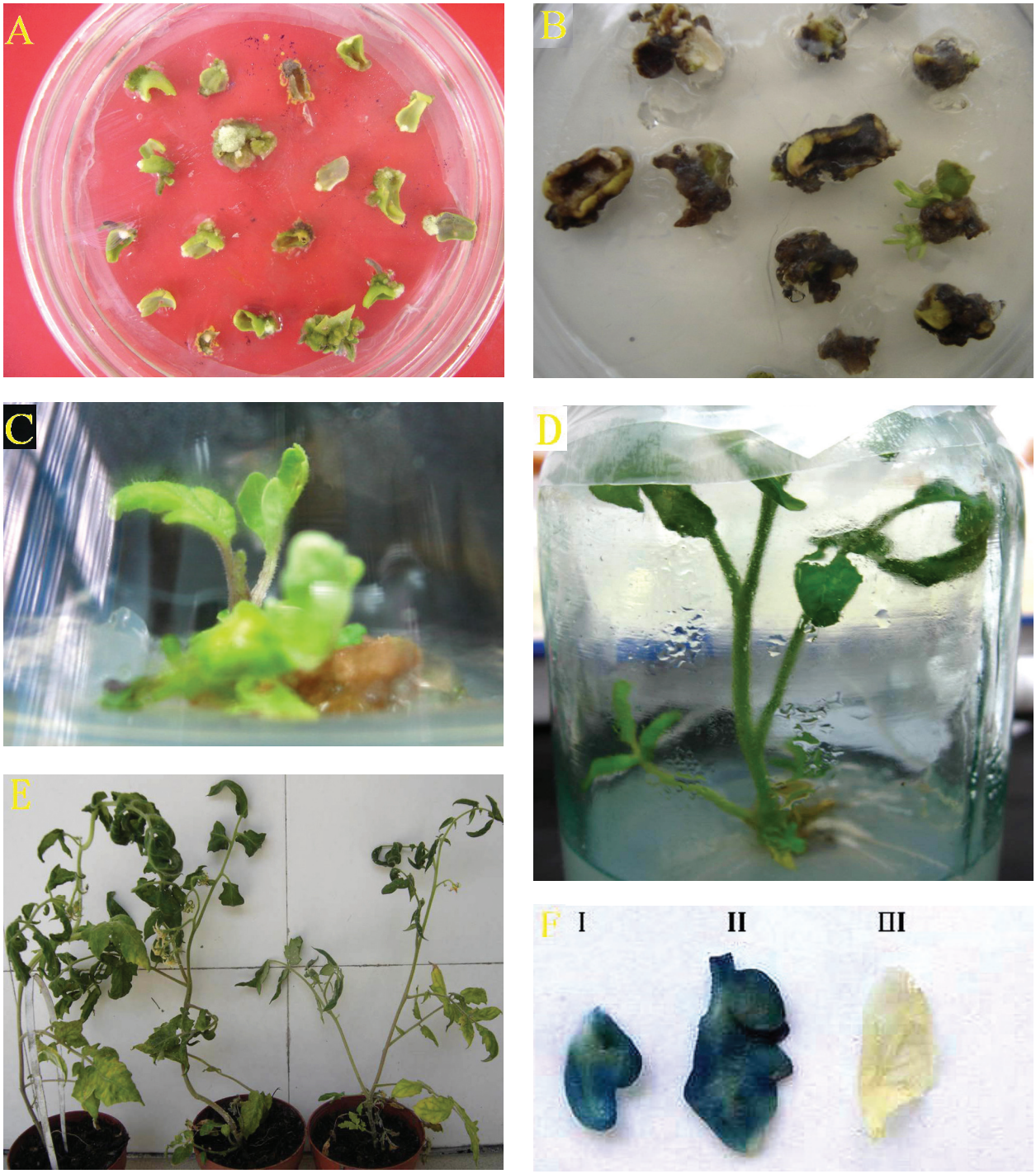

Figure 3. Transgenic tomato plants obtained after *Agrobacterium*-mediated transformation; (A) – callus and shoots began to appear at the wounded sites of the cotyledon explants after 2 weeks of selection, (B) – shoots regenerated from callus 4 weeks later, (C) – shoots were transferred onto MS medium for elongation, (D) – shoots growing well on the rooting medium, (E) – mature plants transplanted in plastic pots, (F) – GUS staining of the transgenic T_0 plants: I–II transgenic tomato plants; III non-transgenic tomato plants (negative control)

the transgenic plants while it was absent in the untransformed wild-type tomato plants (negative control). The length (495 bp) of the PCR amplification fragment was identical with that amplified from pCAMBIA-1301 plasmid (positive control). The results primarily verified that the target gene

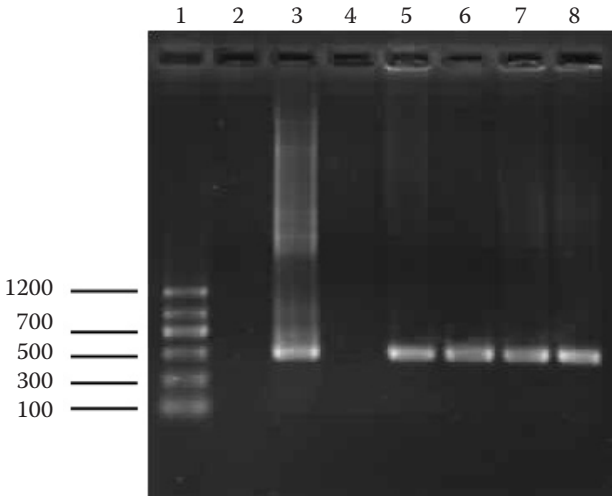

Figure 4. PCR analysis of transgenic tomato plants; 1 – DNA marker II (DL 1200 bp); 2,4 – negative control; 3 – positive control; 5–8 – transgenic tomato plants

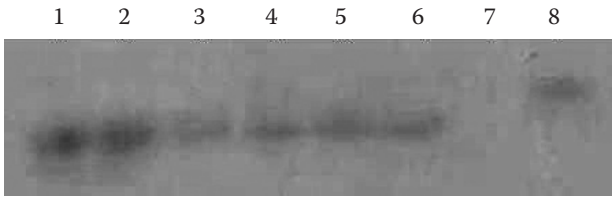

Figure 5. PCR-Southern blot analysis of transgenic tomato plants; 1–6 – transgenic tomato plants; 7 – non-transgenic tomato plant (negative control); 8 – enzyme shearing of pBRSAg with *Xba* I/*Kpn* I (positive control)

was integrated into the genomic DNA of transformed tomato plants (Figure 4).

The PCR products after 1% gel separation were blotted onto nylon membranes. The plasmid DNA containing the *HBsAg* gene was labelled with DIG and used for the hybridization with the amplified PCR products to confirm the success of transformation. The transformed plants gave the same hybridization spots as did the positive control, whereas the untransformed plants showed no detectable hybridization signal (Figure 5). The results of PCR-Southern blot analysis clearly demonstrated the presence of the target gene in the tomato plants.

CONCLUSION

Plants can be used as the most effective systems for the production of therapeutic proteins, including recombinant vaccines. In our study, tomato cotyledon explants were transformed with the plant expression vector pBRSAg harbouring *HBsAg* gene via *Agrobacterium*-mediated approach. The transgenic nature of the plants was confirmed by GUS staining, PCR and PCR-Southern analysis. A number of factors which are important for the consistent production of transgenic tomato plants including *Agrobacterium* strains, infection time, co-cultivation time, *Agrobacterium* concentrations and acetosyringone concentrations were evaluated. The present results demonstrated the feasibility and effectiveness of *A. tumefaciens* strain LBA4404 harbouring plasmids with *HBsAg* gene under the optimized conditions for tomato transformation. This study would lay the foundation for the development of a new type of plant-derived hepatitis B vaccine or other edible vaccines and promote their practical application. However, additional study would be required to be carried out. The transformants should be further confirmed by Southern blot and Northern blot, which are superior to PCR and PCR-Southern. The target gene/gene of interest (GOI) expression should be detected by Western blot while the amount of HBsAg protein should be detected by ELISA. In addition, more experiments are needed to confirm the inheritance of transgenes in successive generations (T_1, T_2) and the mode of GOI heritability.

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